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Demand-oriented riboflavin supply of organic broiler using a feed material from fermentation of *Ashbya gossypii*

C. Lambertz [a,*], J. Leopold [a], S. Ammer [b,c], F. Leiber [b], B. Thesing [c], C. Wild [d], K. Damme [d]

[a] *Research Institute of Organic Agriculture (FiBL), Kasseler Strasse 1a, 60486 Frankfurt am Main, Germany*
[b] *Research Institute of Organic Agriculture (FiBL), Ackerstrasse 113, 5070 Frick, Switzerland*
[c] *Department of Animal Sciences, Georg-August-University, Albrecht-Thaer-Weg 3, 37075 Göttingen, Germany*
[d] *Poultry Competence Centre of the Bavarian Institute for Agriculture, Mainbernheimer Strasse 101, 97318 Kitzingen, Germany*

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Alternatives to riboflavin (vitamin B_2) production by recombinant microorganisms are needed in organic poultry production, but are cost-intensive, so that a demand-oriented riboflavin supply is necessary. Details on the riboflavin requirements of organic poultry are not available. A feed material with high native riboflavin content from fermentation of the filamentous fungus *Ashbya gossypii* was studied. Two runs with 800 Ranger Gold™ broilers each (40 pens with 20 animals) were conducted. The fattening period was divided into **starter (S)**, **grower (G)** and **finisher (F)** stage. In the first run, a basal diet without riboflavin supplementation (NATIVE; 3.27, 3.50 and 3.16 mg riboflavin/kg DM in S, G and F) was compared to diets with supplementation at low (LOW; 5.30, 4.85 and 5.19 mg/kg in S, G and F), medium (MEDIUM; 7.56, 6.88 and 7.56 mg/kg in S, G and F) and high (HIGH; 10.38, 9.14 and 9.93 mg/kg in S, G and F) dosage. In the second run, different combinations of low and medium riboflavin supplementation were used in S, G and F diets: S-LOW (4.50 mg riboflavin/kg DM), G-MEDIUM (6.66 mg/kg), F-MEDIUM (5.71 mg/kg) (Treatment A), S-LOW (4.50 mg riboflavin/kg DM); G-LOW (4.92 mg/kg), F-LOW (4.01 mg/kg) (Treatment B); S-MEDIUM (6.37 mg/kg), G-MEDIUM (7.37 mg/kg), F-MEDIUM (5.07 mg/kg) (Treatment C); S-MEDIUM (6.37 mg/kg), G-LOW (5.28 mg/kg), F-LOW (4.22 mg/kg) (Treatment D). Body weight, feed and water consumption were recorded weekly, health and welfare indicators were scored bi-weekly. Slaughter traits were assessed for five males and females per pen. In the first run, NATIVE animals showed symptoms of riboflavin deficiency and lower live weights in the second week of age. Riboflavin contents of this group were increased to avoid further deficiency and recovery was observed. Feed conversion was better in HIGH (2.07) compared with NATIVE and LOW (2.11). At slaughter, treatments differed neither for foot pad dermatitis nor plumage cleanliness. In the second run, daily weight gains did not differ between treatments in any of the weeks. Feed conversion ranged between 1.99 and 2.04. Riboflavin deficiency was not observed in the second run, while treatment D showed superior economic efficiency. In conclusion, native contents of feed components (3.27 mg/kg DM) were not sufficient to meet the riboflavin demand and a total content of 4.50 mg/kg DM was identified as safe lower threshold. The levels rather according to commercial recommendations were not additionally beneficial to performance and health.

Implications

Currently, there is no product on the market, which is suitable for riboflavin (vitamin B_2) supplementation in organic diets for monogastrics, because EU organic regulations do not allow the use of products from genetically modified organisms. As shown in the present study, native contents of feed materials are however not sufficient to meet the demand of the animals. A feed material with high native riboflavin content from fermentation of the filamentous fungus *Ashbya gossypii* produced according to organic regulations proved its feasibility to be used in broiler diets.

Introduction

Currently, there is no feed additive for riboflavin supplementation of poultry diets available on the market that meets the European organic regulations (EC, 2007, 2008). Even though the riboflavin demand of poultry raised under organic conditions is unknown, native contents of feed components are generally too low to meet the requirements of young growing chicken (NRC, 1994; McDowell, 2008). The components most frequently used contain highly variable riboflavin contents. Recently, riboflavin contents of 0.62 to 1.58 mg/kg DM in cereals and

* Corresponding author.
E-mail address: christian.lambertz@fibl.org (C. Lambertz).

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1.00 to 3.84 mg/kg DM in legumes were analysed (Witten and Aulrich, 2018, 2019). Recommendations that are outdated and were not derived from study conditions that represent the current production conditions vary between 2.6 and 3.6 mg/kg (Ruiz and Harms, 1988; Chung and Baker, 1990; NRC, 1994; Roth-Maier and Kirchgeßner, 1997). The latter recommendation level was, however, not sufficient for fast-growing broilers in the study of Olkowski and Classen (1998). This undermines the necessity to update vitamin recommendations to current performances. Breeding companies recommend much higher dosages, e.g. 8.6 mg/kg starter feed for fast-growing genotypes (Aviagen, 2019). Compared to vitamin production at industrial scale using genetically modified organisms (**GMO**), production without modification is much more cost-intensive. It is therefore economically warranted to use alternative products at demand-oriented levels.

Deficiency in riboflavin, however, has to be avoided as it causes serious health problems and also leads to decreased performance (Ogunmodede, 1977; Chung and Baker, 1990). Severe deficiency frequently results in nervous malformations, footpad dermatitis and 'curled-toe paralysis' (Wyatt et al., 1973; Johnson and Storts, 1988; Cai et al., 2006; Shepherd and Fairchild, 2010). In the second week of age broiler chicken are most sensible to riboflavin deficiency (Olkowski and Classen, 1998). Deyhim et al. (1992) reported the NRC recommendation to be satisfactory when growing broilers are fattened for four weeks but found benefits of greater riboflavin supplementation when animals were raised for eight weeks. Consequently, vitamin requirements in organic livestock production may differ from those in conventional systems, but no specific data are currently available.

Concluding, alternative sources of riboflavin to be used in diets for monogastric animals are needed for the organic sector and other label programmes that are based on non-GMO materials in feed. In a recent study, Lambertz et al. (2019) studied the use of a fermentation suspension with high native riboflavin content from fermentation of the filamentous fungus *A. gossypii* produced according to organic regulations. Results indicated that the fermentation suspension can be used as alternative to riboflavin produced from GMO in broiler feeding. From a practical point of view, a dried product is beneficial to the use of the suspension. Therefore, diets without and with the dried fermentation product at different dosages were used in order to derive advices for a demand-oriented riboflavin supplementation of organic broilers.

Material and methods

Birds and housing

The study consisted of two runs and was conducted at the Poultry Competence Centre of the Bavarian Institute for Agriculture, Germany. In each run, 800 mixed-sex one-day old Ranger Gold™ (Aviagen Epi GmbH, Cuxhaven, Germany) broilers were raised under floor husbandry conditions until 55 days of age. Animals were randomly allocated to 40 groups of 20 animals. The stocking density was four animals/m^2. Feed and water were available ad libitum. Vaccinations against infectious bronchitis (day 1) and against infectious bronchitis and Newcastle's disease (day 16) were done.

Diets

The fattening period was divided into starter (**S**, 14 days), grower (**G**, 21 days in run 1 and 28 days in run 2) and finisher (**F**, 20 days in run 1 and 13 days in run 2) stage. A basal diet of organic feed components was produced by a commercial feed mill (Kaisermühle Gänheim Otmar Kaiser GmbH, Arnstein-Gänheim, Germany). The premix was formulated without riboflavin supplementation. The composition of the basal diet for S, G and F diets was the same for the first and the second run (Table 1). Prior to feed production of each run, the vitamin B_2 content of the single feed components was analysed by HPLC (method DIN EN 14152:2006) by LUFA-ITL GmbH (Kiel, Deutschland) and calculated as riboflavin content (Table 1).

The dietary treatments were prepared by supplementing a feed material with a high native riboflavin content produced by fermentation of the yeast-like fungus *A. gossypii*. The product named EcoVit R Powder of the company Agrano GmbH & Co. KG (Riegel am Kaiserstuhl, Germany) is registered as feed material in the EU Feed Materials Register (008290-EN). Furthermore, it is produced according to organic standards and therefore certified organic. An *A. gossypii* strain from a publicly available collection was used. In an aseptic fermentation process a substrate of organically certified sugar, oil and nitrogen was fermented. The final product was not purified. It was thermally treated to inactivate *A. gossypii*, dried by evaporation and subsequent drum drying and grinded. The powder is stable at 22 °C for at least 6 months. The product does not contain any ingredients subject to a specific labelling regarding genetically modified organisms in accordance with the European Regulation (EC) No. 1829/2003 and No. 1830/2003. The powder was added to the feed mixer at the time the premix, herbal mixture, calcium carbonate and potassium chloride were added. After mixing the feed was pelleted and mashed.

In the first run, a basal diet without supplementation of the fermentation product (**NATIVE**) was compared to three diets with increasing dosage of the product (**LOW**, **MEDIUM**, **HIGH**). The NATIVE treatment had a riboflavin content of 3.27, 3.50 and 3.16 mg/kg DM for S, G and F diets. The riboflavin content of the S, G and F diet was 5.30, 4.85 and 5.19 mg/kg DM for S, G and F diet in LOW, 7.56, 6.88 and 7.56 mg/kg DM for S, G and F in MEDIUM and 10.38, 9.14 and 9.93 mg/kg for S, G and F in HIGH treatment. The nutrient composition analysed according to standard methods (Method VO(EG) 152/2009) by LUFA-ITL GmbH (Kiel, Deutschland) of the four dietary treatments is presented in Table 2. The riboflavin-content of single feed components was analysed by HPLC (method DIN EN 14152:2006) and calculated as riboflavin content.

In the second run, four dietary treatments were compared. Each treatment was used in 10 groups of 20 animals. For S, G and F stage, diets with low and medium riboflavin content each were formulated. Based on the results of the first run, which showed that in the starter period, the NATIVE treatment was not sufficient to meet the demand of the animals while the HIGH supplementation was not beneficial, the two S diets LOW and MEDIUM of the first run were used. The riboflavin content of the S diet was analysed prior to the start of the run, which started three weeks after the first ended. G and F diets were produced at the start of the second run. The nutritional composition of the dietary treatments is given in Table 3. Treatments A to D received the following S, G and F diets: treatment A: S-LOW (4.50 mg riboflavin/kg DM), G-MEDIUM 1 (6.66 mg/kg), F-MEDIUM 1 (5.71 mg/kg); treatment B: S-LOW (4.50 mg riboflavin/kg DM), G-LOW 1 (4.92 mg/kg), F-LOW 1 (4.01 mg/kg); treatment C: S-MEDIUM (6.37 mg/kg), G-MEDIUM 2 (7.37 mg/kg), F-MEDIUM 2 (5.07 mg/kg); treatment D: S-MEDIUM (6.37 mg/kg), G-LOW 2 (5.28 mg/kg), F-LOW 2 (4.22 mg/kg).

Body weight was recorded weekly and uniformity was calculated using individual body weights at the end of the fattening period. Mortality was recorded daily. Feed consumption was measured at weekly intervals at pen level by subtracting feed residuals from offered feed amounts. Each pen was equipped with a recorder that measured water consumption at pen level. With the data, the water: feed ratio was calculated. According to the Welfare Quality® Assessment protocol for poultry (Welfare Quality®, 2009), 5 randomly selected animals of each pen were subjected bi-weekly (day 14, 28, 42, 54) to scoring of plumage cleanliness (score 0 = clean to 3 = heavily soiled), foot pad dermatitis (score 0 = no foot pad dermatitis to 2 = severe foot pad dermatitis) and hock burn (score 0 = no evidence of hock burn to score 2 = evidence of hock burn). Additionally, litter was scored weekly according to the same protocol (score 0 = completely dry and flaky litter, moves easily to score 4 = litter sticks to boots once the cap or compacted crust is broken).

Slaughter parameters were recorded in 200 animals per run (25 randomly selected males and 25 females of each treatment). For these

Table 1
Composition of the starter, grower and finisher diets for broilers and riboflavin content of the feed components in the first and second run.

Component	Fattening stage			Riboflavin content of feed components			
				Run 1		Run 2	
	Starter diet (%)	Grower diet (%)	Finisher diet (%)	DM (%)	Riboflavin (mg/kg FM)	DM (%)	Riboflavin (mg/kg FM)
Soy cake (pellet)	34.9	25.5	27.2	89.6	3.00	89.6	3.03
Wheat (whole grain)	24.5	28.1	27.8	87.3	1.00	87.3	0.88
Maize (whole grain)	15.0	15.9	16.5	88.8	0.90	88.8	0.86
Wheat bran	-	8.0	8.0	86.1	2.50	86.1	2.46
Wheat gluten feed (pellet)	9.1	-	-	92.2	3.80	92.2	3.27
Peas (whole bean)	-	7.6	-	87.1	1.70	87.1	1.53
Maize gluten feed (pellet)	4.5	4.6	4.6	92.1	3.00	92.1	2.19
Sesame cake (pellet)	-	4.0	4.0	91.7	5.10	91.7	5.09
Grass meal (powder)	-	-	3.5	94.5	9.00	94.5	8.18
Sunflower oil	2.5	2.5	2.5	99.9	[1]	99.9	[1]
Beer yeast (powder)	2.5	-	-	93.4	21.6	NA	NA
Sunflower cake (pellet)	1.9	-	-	93.4	3.31	NA	NA
$CaCO_3$	1.8	1.6	1.5	NA	NA	NA	NA
Glucose	1.0	-	-	NA	NA	NA	NA
Monocalcium phosphate	0.8	1.2	1.4	NA	NA	NA	NA
Premix[2]	0.6	0.5	0.5	96.2	[1]	96.2	[1]
Casein	0.5	-	-	92.6	6.4	NA	NA
Diatomaceous earth	0.3	0.3	0.3	NA	NA	NA	NA
Salt	0.1	0.2	0.2	NA	NA	NA	NA
Oregano	0.03	0.03	0.03	NA	NA	NA	NA

FM = fresh matter.
NA = not analysed.
[1] Below the lower limit of detection.
[2] The premix was prepared by the company Miavit GmbH (Essen, Germany) without riboflavin supplementation.

animals, dressing percentage and weights of abdominal fat, liver, heart and gizzard were recorded. In addition, liver color (score 0 = dark red to score 2 = yellowish, disrupted structure) was scored. With the data collected, the European broiler index (**EBI**) was calculated according to Marcu et al. (2013): Daily weight gain (g) x survival rate (%)/feed conversion (kg feed/kg body weight gain) x 10.

Statistical analysis

The two runs were analysed separately using the software package SAS, version 9.3 (Statistical Analysis Systems, Cary, North Carolina, USA). Performance and slaughter traits were analysed applying the ANOVA (GLM procedure) with the dietary treatment as fixed effect and pen as random effect. Pen was considered as the statistical unit. Schematic boxplots and UNIVARIATE procedure were used to test normal distribution of data. The Tukey-Kramer test was used to compare differences between treatments with α set at 0.05. Results are presented as least squares means ± SE. A chi-square test was applied for data of footpad dermatitis, feather condition, hock burn and liver color scoring to determine differences between dietary treatments with α set at 0.05.

Results

Run 1

Animals of the NATIVE treatment showed symptoms of riboflavin deficiency in the second week of age. When the first behavioural disorders (agitation disorders, lameness, walking on joints) were observed six affected broilers (3 males and 3 females) were subjected to inspection by the Bavarian Animal Health Services (Poing, Germany). At good nutritional status and no further organ disorders, mild to

Table 2
Composition of the dietary treatments for broilers and the *A. gossypii* fermentation product in the first run.

Nutrient	Starter diet				Grower diet				Finisher diet				Fermentation product
	NATIVE[1]	LOW	MEDIUM	HIGH	NATIVE	LOW	MEDIUM	HIGH	NATIVE	LOW	MEDIUM	HIGH	
DM (g/kg)	886	888	884	882	882	883	883	885	886	887	888	888	945
Riboflavin (mg/kg DM)	3.27	5.30	7.56	10.38	3.50	4.85	6.88	9.14	3.16	5.19	7.56	9.93	7 404
Crude protein (g/kg DM)	279	281	286	289	249	246	245	246	237	239	239	236	252
Ether extract (g/kg DM)	99	98	98	97	91	67	91	94	88	91	88	95	110
Crude fiber (g/kg DM)	43	44	50	43	45	44	43	47	49	51	50	50	25
Nitrogen-free extracts (g/kg DM)	505	505	493	496	537	566	544	536	550	545	553	547	521
Saccharose (g/kg DM)	56	58	59	62	44	45	47	47	45	44	44	41	220
Starch (g/kg DM)	314	306	305	301	376	379	387	382	404	401	405	397	41
Crude ash (g/kg DM)	75	75	71	71	72	75	73	76	77	75	72	75	159
ME (MJ/kg DM)	13.6	13.5	13.7	13.7	13.9	13.0	14.0	14.0	14.0	14.1	14.1	14.1	11.1
Amino acids													
Lysine (g/kg DM)	13.3	14.1	13.5	13.4	11.6	11.6	11.3	11.6	10.9	10.5	10.6	9.9	7.4
Methionine (g/kg DM)	4.4	4.4	4.4	4.6	4.2	4.1	4.1	4.0	4.1	4.0	4.1	4.1	2.1
Cysteine (g/kg DM)	4.5	4.6	4.9	4.6	4.2	4.1	4.2	4.2	4.1	4.0	4.1	4.2	3.4
Threonine (g/kg DM)	10.0	10.4	10.2	10.4	9.0	8.8	8.7	8.7	8.6	8.4	8.6	8.4	8.1

ME = metabolizable energy.
[1] The riboflavin content of the NATIVE treatment was increased to 6.63 mg/kg DM in the second week of age because of deficiency symptoms. The riboflavin content of the grower diet was increased to 5.39 mg/kg and of the finisher diet to 5,74 mg/kg.

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Table 3
Nutrient composition of the dietary treatments for broilers and the *A. gossypii* fermentation product in the second run.

Nutrient	Starter (S) diet[1]		Grower (G) diet				Finisher (F) diet			
	S-LOW	S-MEDIUM	G-LOW 1	G-LOW 2	G-MEDIUM 1	G-MEDIUM 2	F-LOW 1	F-LOW 2	F-MEDIUM 1	F-MEDIUM 2
DM (g/kg)	888	886	889	892	889	887	890	891	890	891
Riboflavin (mg/kg DM)	4.50	6.37	4.92	5.28	6.66	7.37	4.01	4.22	5.71	5.07
Crude protein (g/kg DM)	286	286	245	240	242	248	237	238	236	237
Ether extract (g/kg DM)	96	97	78	80	78	80	74	77	69	79
Crude fiber (g/kg DM)	43	44	50	43	45	44	43	47	49	51
Nitrogen-free extracts (g/kg DM)	505	505	493	496	537	566	544	536	550	545
Saccharose (g/kg DM)	53	53	55	55	55	54	54	53	53	54
Starch (g/kg DM)	289	26	370	370	323	368	377	386	391	384
Crude ash (g/kg DM)	74	71	73	72	74	73	73	73	73	73
ME (MJ/kg DM)	13.6	13.7	13.8	13.8	13.8	13.9	13.7	13.9	13.7	13.9
Amino acids										
Lysine (g/kg DM)	13.1	12.9	11.0	11.7	11.3	10.9	10.7	10.7	10.8	10.8
Methionine (g/kg DM)	4.6	4.5	3.8	4.0	4.0	3.8	3.9	4.0	3.9	4.0
Cysteine (g/kg DM)	4.6	4.6	4.0	4.3	4.3	6.9	4.3	4.3	4.3	4.3
Threonine (g/kg DM)	10.6	10.4	8.5	8.9	8.9	8.5	8.4	8.5	8.5	8.6

ME = metabolizable energy.
[1] Starter diets were the same as in the first run with riboflavin contents of 5.30 mg/kg DM (LOW) and 7.56 mg/kg DM (MEDIUM) analysed immediately after feed mixing.

moderately severe subacute degenerations of the sciatic nerve were found in all examined animals. To avoid further disorders, the riboflavin content of the NATIVE treatment was increased to 6.63 mg/kg DM by mixing the four dietary treatments. Those of the G and F diets were increased to 5.39 mg/kg and 5.74 mg/kg, respectively. After the riboflavin content was adjusted no more behavioural disorders indicating riboflavin deficiency were observed in the NATIVE treatment.

Fig. 1 A presents the daily weight gains per week during the fattening period of the first run. Differences between treatments were observed in the second week. With 22.7 g the NATIVE animals had a lower daily weight gain compared with the other treatments that ranged between 24.7 and 26.2 g ($P < 0{,}05$). The greatest daily weight gains of more than 65 g were observed in all groups in the sixth and seventh week. Averaged over the whole fattening period daily gains ranged between 45.8 and 47.5 g ($P > .05$, Table 4).

With the daily weight gains presented, live weight of the NATIVE treatment (254 g) at two weeks of age, thus before the riboflavin content of this group was adjusted, was lower than of all other treatments (LOW = 278 g; MEDIUM = 272 g; HIGH = 275 g) ($P < .05$). One week later, the NATIVE treatment (485 g) differed from LOW (519 g) ($P < .05$), but not from both other treatments (504 g) ($P > .05$). In the subsequent week, differences between treatments were not observed. At the end of the fattening period, live weights were 2 518 g, 2 610 g, 2 585 g and 2 587 g for NATIVE, LOW, MEDIUM and HIGH ($P > .05$, Table 4). Uniformity at the end of the fattening period was greatest in NATIVE (63.6%) and was followed by LOW (53.3%), HIGH (50.0%) and MEDIUM (47.5%).

Feed consumption over the whole fattening period did not differ between treatments in a range between 5 129 and 5 412 g per animal ($P > .05$, Table 4), whereas feed conversion was better in HIGH (2.07) compared with NATIVE and LOW (2.11) ($P < .05$). NATIVE animals consumed less water than LOW animals ($P < .05$), while water:feed ratio did not differ between treatments ($P > .05$). Mortality was greater in NATIVE (4.5%) than in LOW and HIGH (2.5%) as well as MEDIUM (1.5%), while the rate within the first week of age was below 1% in all groups. Three percent of the losses in the NATIVE treatment occurred during the second week of age. EBI was lower for NATIVE (211 points) than in the other treatments (224 to 226 points) ($P < .05$), which can be explained by the differences in mortality rate and feed conversion rate.

Body and slaughter weight of the animals that were subjected to assessment of slaughter traits did not differ between treatments ($P < .05$; Table 4). Dressing was approximately 1% greater in MEDIUM and HIGH than in NATIVE ($P < .05$). Breast and thigh percentage did not vary ($P > .05$), while percentage of wings was lower in LOW and HIGH than in NATIVE ($P < .05$). NATIVE had a lower carcass percentage

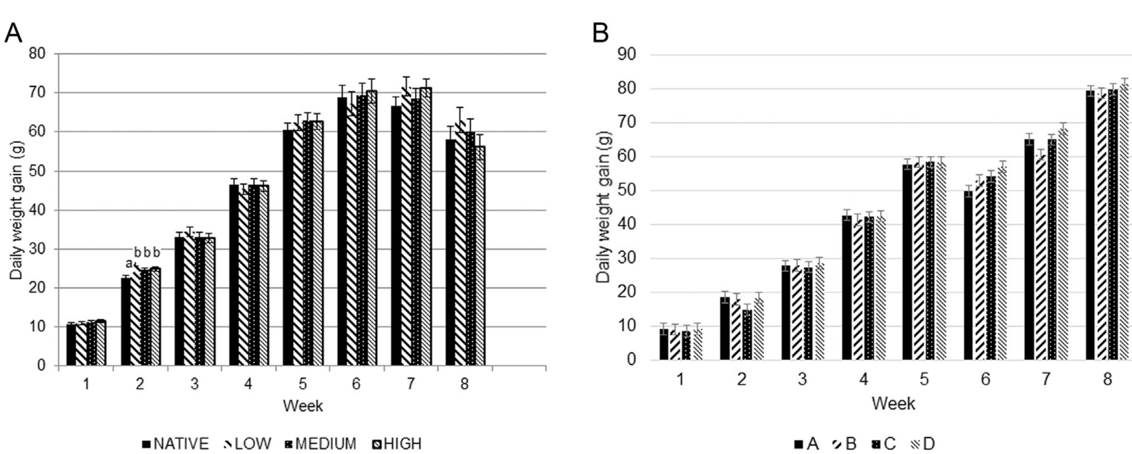

Fig. 1. Daily weight gain per week during the fattening period of broilers fed with a basal diet without riboflavin supplementation (NATIVE) or with riboflavin from *A. gossypii* fermentation at low (LOW), medium (MEDIUM) and high (HIGH) dosage in the first run (n = 200 animals per treatment, Fig. 1 A) and with treatments A to D (see Table 6 for dietary treatments) in the second run (n = 200 animals per treatment, Fig. 1 B). The riboflavin content of the NATIVE treatment was increased to 6.63 mg/kg DM in the second week because of deficiency symptoms.

Table 4
Performance and slaughter traits for broilers fed with a basal diet without riboflavin supplementation (NATIVE) or with riboflavin from *A. gossypii* fermentation at low, medium and high dosage in the first run.

Item	Dietary treatment				SE
	NATIVE[1]	LOW	MEDIUM	HIGH	
Performance traits (n = 200)					
Body weight at day 54 (g)	2 518.4	2 610.1	2 584.9	2 587.2	34.0
Daily weight gain (g)	45.8	47.5	47.1	47.1	0.7
Feed consumption (g/animal)	5 219	5 412	5 311	5 253	77.1
Feed conversion rate (kg feed/kg gain)	2.11[b]	2.11[b]	2.09[ab]	2.07[a]	0.02
Water consumption (ml)	10 947[b]	11 326[a]	11 034[ab]	11 005[ab]	142
Water:feed ratio (ml/g)	2.10	2.10	2.08	2.10	0.33
EBI (points)	211[b]	224[ab]	226[a]	226[a]	4
Slaughter traits (n = 50)					
Body weight at day 54 (g)	2 552	2 611	2 569	2 566	35.1
Slaughter weight (SW, g)	1 881	1 937	1 928	1 925	29.0
Dressing (%)	73.72[b]	74.22[ab]	74.95[a]	74.95[a]	0.3
Breast (% of SW)	25.44	25.45	25.80	25.67	0.27
Thigh (% of SW)	30.12	30.33	30.35	30.21	0.24
Wings (% of SW)	11.18[a]	10.82[b]	11.01[ab]	10.89[b]	0.09
Carcass (% of SW)	1.50[c]	1.77[a]	1.53[bc]	1.75[ab]	0.08
Abdominal fat (% of SW)	2.93[a]	2.91[a]	2.70[b]	2.59[b]	0.05
Liver (% of SW)	1.45[a]	1.34[b]	1.19[c]	1.26[bc]	0.04
Heart (% of SW)	0.54[a]	0.57[a]	0.47[b]	0.48[b]	0.01
Gizzard (% of BW)	1.45[a]	1.34[b]	1.19[c]	1.26[bc]	0.04

[a,b,c] Different superscripts within rows indicate significant differences between treatments at P<0.05.
SE = standard error.
EBI = European broiler index: daily weight gain (g) x survival rate (%)/feed conversion (kg feed/kg body weight gain) x 10.
[1] The riboflavin content of the NATIVE treatment was increased to 6.63 mg/kg DM in the second week of age because of deficiency symptoms. The riboflavin content of the grower diet was increased to 5.39 mg/kg and of the finisher diet to 5,74 mg/kg.

compared with LOW and HIGH (P < .05). Abdominal fat had a greater percentage in NATIVE and LOW than in both other treatments (P < .05). Treatment NATIVE had a greater percentage of liver, heart and gizzard than MEDIUM and HIGH (P < .05). The frequency of livers scored with 2 (yellowish color, disrupted structure) was highest in LOW animals (12%; Table 5). The HIGH treatment had the highest frequency of score 0 (dark red color).

At the assessments during the fattening period, incidences of foot pad dermatitis were not found at any of the assessment dates. For plumage cleanliness, differences were only recorded at day 42 with NATIVE and HIGH having less than 20% of the animals scored with 2 and both other with 30% and more, though score 3 was not found in any of the animals ($\chi^2 = 10.93$; $P < .05$). At slaughter, neither for foot pad dermatitis nor plumage cleanliness treatments differed ($P > .05$; Table 5), whereas only two animals at all were observed with foot pad dermatitis score 1. Hock burns were not recorded at slaughter. Until 7 weeks of age, litter of all pens was scored 0. The average score increased to 1.7 in HIGH and 1.6 in all other treatments at slaughter ($P > .05$).

Run 2

The daily weight gains did not differ between treatments in any of the weeks during the fattening period of the second run (P < .05; Fig. 1 B). Contrary to the first run, the greatest daily weight gains of 78.6 to 81.2 g were reached in the last week before slaughter (week 8). Averaged over the whole fattening period, daily weight gains of 42.6 g, 42.2 g, 42.9 g and 44.2 g for treatments A, B, C and D, respectively ($P > .05$; Table 6). At the end of the fattening period, D-animals reached 2 429 g, while the other treatments ranged between 2 316 and 2 358 g ($P > .05$). Treatment D (51.9%) had the lowest uniformity at the end of the fattening period followed by treatments B (54.4), C (55.1%) and A (57.5%).

Feed consumption varied between 4 691 and 4 750 g per animal without a difference between treatments (P > .05; Table 6). Feed conversion was lower in D (1.99 kg/kg) than in the other treatments (2.04 to 2.06 kg/kg) ($P < .05$). Without a difference between treatment ($P < .05$) the animals consumed 10 585 to 10 911 ml water throughout the fattening period. The water:feed ratio ranged between 2.23 and 2.33 ($P > .05$). Mortality was summed up to 0.5% in A and B and 1.5% in C and D, whereas the rate observed in the first week of age was 0.0% in A and B, 1.0% in C and 0.5% in D.

Table 5
Chi-square test of liver color, foot pad and plumange cleanliness scoring at slaughter of broilers fed with a basal diet without riboflavin supplementation (NATIVE) or with riboflavin from *A. gossypii* fermentation at low, medium and high dosage of the first run (n = 50 animals per treatment)[1, 2, 3 and 4].

Item	Dietary treatment			
	NATIVE[1]	LOW	MEDIUM	HIGH
Liver color[2]				
0	19	18	28	32
1	30	26	22	17
2	1	6	0	1
$\chi = 20.71$; P < 0.01				
Food pad dermatitis[3]				
0	48	50	50	50
1	2	0	0	0
2	0	0	0	0
$\chi = 6.06$; P > 0.05				
Plumage cleanliness[4]				
0	8	6	6	9
1	25	23	24	28
2	17	21	20	13
3	0	0	0	0

[1] The riboflavin content of the NATIVE treatment was increased to 6.63 mg/kg DM in the second week of age because of deficiency symptoms. The riboflavin content of the grower diet was increased to 5.39 mg/kg and of the finisher diet to 5,74 mg/kg.
[2] Score 0 = dark red to score 2 = yellowish, disrupted structure.
[3] Score 0 = no foot pad dermatitis to score 2 = severe foot pad dermatitis.
[4] Score 0 = clean to 3 = heavily soiled.

Table 6
Performance and slaughter traits of broilers fed with different riboflavin contents during starter, grower and finisher phase of the second run.

Item	Dietary treatment[1]				SE
	A	B	C	D	
Performance traits (n = 200)					
Body weight at day 54 (g)	2 341	2 316	2 358	2 429	37.3
Daily weight gain (g)	42.6	42.2	42.9	44.2	0.7
Feed consumption (g/animal)	4 692	4 691	4 733	4 750	82.6
Feed conversion rate (kg feed/kg gain)	2.04[b]	2.06[b]	2.04[b]	1.99[a]	0.02
Water consumption (ml)	10 911	10 853	10 700	10 570	136
Water:feed ratio (ml/g)	2.33	2.32	2.27	2.23	0.03
EBI (points[3])	212[ab]	207[b]	211[b]	223[a]	4
Slaughter traits (n = 50)					
Body weight at day 54 (g)	2 385	2 355	2 324	2 449	33.7
Slaughter weight (SW, g)	1 715[ab]	1 713[ab]	1 686[a]	1 809[b]	27.0
Dressing (%)	71.88[a]	72.69[a]	72.52[a]	73.89[b]	0.27
Breast (% of SW)	24.44	24.46	24.38	25.00	0.23
Thigh (% of SW)	31.33	31.37	31.48	31.12	0.18
Wings (% of SW)	11.32	11.29	11.41	11.21	0.07
Carcass (% of SW)	27.39[a]	27.34[ab]	27.12[ab]	26.80[b]	0.15
Abdominal fat (% of SW)	1.30	1.45	1.37	1.49	0.07
Liver (% of SW)	3.23[a]	3.06[a]	3.10[a]	2.82[b]	0.07
Heart (% of SW)	0.50[a]	0.48[ab]	0.48[ab]	0.46[b]	0.01
Gizzard (% of BW)	1.34[ab]	1.42[a]	1.38[ab]	1.24[b]	0.04

SE = standard error.
EBI = European broiler index: daily weight gain (g) x survival rate (%)/feed conversion (kg feed/kg body weight gain) x 10.
[a,b,c] Different superscripts within rows indicate significant differences between treatments at P<0.05.
[1] A, 4.50 mg riboflavin/kg DM in starter, 6.66 mg/kg in grower and 5.71 mg/kg in finisher diet; B, 4.50 mg riboflavin/kg DM in starter, 4.92 mg/kg in grower and 4.01 mg/kg in finisher diet; C, 6.37 mg/kg riboflavin/kg DM in starter, 7.37 mg/kg in grower and 5.07 mg/kg in finisher diet; D, 6.37 mg/kg riboflavin/kg DM in starter, 5.28 mg/kg in grower and 4.22 mg/kg in finisher diet.

Animals of treatment D (223 points) had a higher EBI-value than all other groups that ranged between 207 and 212 points ($P < .05$; Table 6).

Body weight of the animals that were assessed for slaughter traits did not vary between treatments ($P < .05$), but D animals had a higher slaughter weight than C ($P < .05$; Table 6). Dressing was greater in D than in the other treatments ($P < .05$), while percentages of breast, thigh and wings did not differ ($P > .05$). Percentage of liver, heart and gizzard was lowest in D animals. Liver score did not differ between treatments ($P > .05$), whereas approximately half of the animals were scored with 0 (Table 7).

Foot pad dermatitis did not show any treatment differences throughout the fattening period ($P > .05$). At slaughter, the proportion of animals with score 1 was low in all groups, while score 2 was not recorded (Table 7). Alike, plumage cleanliness did not differ at any of the assessments throughout the fattening period ($P > .05$). Hock burns were not recorded at slaughter. Until 6 weeks of age, litter was scored 1 in all pens. At 7 weeks of age, the average score of treatments A and B increased to 1.8 and of the other two treatments to 1.7 ($P > .05$). In the final week of the fattening period, average scores were 1.5, 1.6, 1.8 and 1.9 for treatments C, B, A and D, respectively ($P > .05$).

Discussion

The present study evaluated a feed material with high native riboflavin content from fermentation of the filamentous fungus *A. gossypii* produced according to organic regulations in slow-growing broilers. This is an alternative source of riboflavin to be used in diets for monogastric animals for the organic sector and other label programmes that are based on non-GMO materials in feed. The aim was to derive advices for a demand-oriented riboflavin supplementation given that high production costs compared to conventional riboflavin production from GMO warrant an adjusted supplementation from an economic point of view without inducing deficiency in this vitamin.

A diet without riboflavin supplementation and only native contents of the organic feed components with a similar riboflavin content than in this study induced deficiency symptoms in slow-growing broilers in the second week of age. Behavioural disorders included agitation disorders, lameness and walking on joints that are typical for the so-called 'curled-toe-paralysis' (Wyatt et al., 1973; Johnson and Storts, 1988; Cai et al., 2006; Shepherd and Fairchild, 2010). In examined animals, mild to moderately severe subacute degenerations of the sciatic nerve supported the assumption of riboflavin deficiency. This also led to a higher mortality rate in the NATIVE treatment during the second week of age. Animals recovered after the riboflavin content in feed was increased. Differences in body weight that were observed in the deficient group at two weeks of age were not found in subsequent weeks. These findings confirm results of Johnson and Storts (1988), who observed recovery of riboflavin-deficient animals after 35 days. It also emphasizes that growth performance is a very sensitive indicator for riboflavin deficiency (Wyatt et al., 1973). Even though, recovery of the NATIVE treatment during the fattening period compensated for the lower growth performance during the first two weeks of age, the differences in dressing and percentage of wings, liver, heart and gizzard to the other groups at slaughter indicates that deficiency during early life affected slaughter parameters. This, however, needs further validation and comparable studies are not available, yet. In this context, determining the riboflavin status in different tissues may also be a valuable indicator beside growth performance to validate thresholds for riboflavin deficiency.

Compared with the previous study (Lambertz et al., 2019), in which the NATIVE diet with a slightly higher riboflavin content (3.39 mg/kg DM) did not induce any sign of riboflavin deficiency in animals of the same genetic, the growth performance in this study was greater and met the performance objectives of the breeding company (Aviagen, 2018). This may be the main explanation for the different observations in both studies. With a broiler diet containing 2.6 mg riboflavin/kg FM, Ruiz and Harms (1988) observed riboflavin deficiency between 10 and 12 days of age, high mortality and poor growth. Roth-Maier and Kirchgeßner (1997) found similar effects in fast-growing Ross broilers that received diets containing 0.65 mg/kg feed of native riboflavin and were not supplemented or with 1 mg riboflavin/kg. The group without riboflavin supplementation reacted with altered feed intake and lower growth after the first week of age. Animals with symptoms had to be excluded from the study at 12 days of age when mortality already reached 20%. The group receiving supplementation of 1 mg/kg also had to be removed from the study at 15 days of age, so that observations of recovery are not available from the study. Other studies with diets containing 1.8 mg/kg (Cai et al., 2006) and 2.6 mg/kg feed (Olkowski and Classen, 1998) also observed signs of riboflavin deficiency in form of leg weakness in the first and nervous malformations in the latter mentioned study. When 2 mg/kg were supplemented signs of deficiency were not recorded by Roth-Maier and Kirchgeßner (1997), so that the authors concluded that the margin between deficient and optimal supply ranges between 1.7 and 2.7 mg/kg. In wide agreement, riboflavin supplementation at 1 to 2 mg/kg was recommended to be sufficient for fast-growing broilers to reach their maximum weight gain (Olkowski and Classen, 1998).

Findings of this as well as both mentioned studies emphasize that even under organic conditions with lower growth performance than under conventional conditions and diets that constitute of more diverse feed components (e.g. legumes) native contents are not sufficient to meet the demand of the animals. The studied fermentation product is therefore one alternative for the organic sector and other label programmes that are based on non-GMO materials in feed to increase riboflavin contents to meet the demand. Confirming other studies (Ruiz and Harms, 1988; Chung and Baker, 1990; Roth-Maier and Kirchgeßner, 1997), broilers are most sensitive to an insufficient riboflavin supply in the first two weeks of age. To minimize the risk of deficiency broiler breeders need sufficient riboflavin supply to ensure an adequate diet to egg transfer (Naber and Squires, 1993), so that chicks hatch with an optimal riboflavin status.

The difference between the riboflavin content of the starter diets in the first and second run may be partly explained by the sensitivity of the riboflavin analysis at low contents (Gul et al., 2014). In a pre-trial

Table 7
Chi-square test of liver color, foot pad and plumage cleanliness scoring at slaughter of broilers fed with different riboflavin contents during starter, grower and finisher phase of the second run (n = 50 animals per treatment).

Item	Dietary treatment[1]			
	A	B	C	D
Liver color[2]				
0	26	30	24	28
1	22	20	25	21
2	2	0	1	1
				$\chi = 3.38$; $P > 0.05$
Food pad dermatitis[3]				
0	50	42	50	46
1	0	8	0	4
2	0	0	0	0
				NA
Plumage cleanliness[4]				
0	29	32	36	27
1	15	11	9	16
2	6	7	5	7
3	0	0	0	0
				$\chi = 4.49$; $P > 0.05$

NA = not analysed.
[1] A, 4.50 mg riboflavin/kg DM in starter, 6.66 mg/kg in grower and 5.71 mg/kg in finisher diet; B, 4.50 mg riboflavin/kg DM in starter, 4.92 mg/kg in grower and 4.01 mg/kg in finisher diet; C, 6.37 mg/kg riboflavin/kg DM in starter, 7.37 mg/kg in grower and 5.07 mg/kg in finisher diet; D, 6.37 mg/kg riboflavin/kg DM in starter, 5.28 mg/kg in grower and 4.22 mg/kg in finisher diet.
[2] Score 0 = dark red to score 2 = yellowish, disrupted structure.
[3] Score 0 = no foot pad dermatitis to score 2 = severe foot pad dermatitis.
[4] Score 0 = clean to 3 = heavily soiled.

of this study, a broiler diet containing conventional riboflavin was sampled five times and analysed for its riboflavin content. At an average content of 7.48 mg/kg FM, the variation coefficient was 10.5% pointing to the sensitivity of the analysis. Losses due to storage time between the two runs of the present study can be excluded because the runs started within 3 months and storage tests of the dried fermentation product at 30 °C for up to 7 months did not result in decreasing riboflavin contents. Exposure to light is most critical for riboflavin, so that a certain safety margin should be considered when diets are formulated and in cases when storage conditions are not optimal. When adjusting the riboflavin content to the demand, it also has to be taken into account that native contents of the feed components are variable, but analysis of each batch currently seems not to be feasible given the cost- and time-intensive riboflavin analysis. The dried product may be included into premixes or added at feed mixing. When used at dosages that are relevant, the nutrient composition of the diet is not affected.

An effect of the different dosages used in the first run on growth performance was not observed, while feed conversion decreased with increasing riboflavin content. In the previous study on the use of the fermentation suspension this was also the case, though growth performance was much lower as the animals did not even reach the body weight after being fattened for one more week assuming that riboflavin demand was lower than in the present one (Lambertz et al., 2019). Increasing riboflavin supplementation from 1 to 10 mg/kg did not affect feed consumption in the study of Olkowski and Classen (1998). Well in agreement with the findings of the present study are also results of Roth-Maier and Kirchgeßner (1997). Increasing the riboflavin supplementation from 2 up to 8 mg/kg did neither affect final body weight nor feed intake and conversion of Ross broilers.

Effects of riboflavin deficiency on animal-based welfare indicators such as plumage cleanliness or footpad dermatitis that were found elsewhere (Shepherd and Fairchild, 2010), could neither be confirmed in the present nor in our previous study (Lambertz et al., 2019). Among others, the greater space allowance and smaller group size than found under practical conditions may have biased results on welfare indicators. Consequently, the litter quality was good during the whole study period. In general, the low prevalence of disorders widely agrees with recent findings of Louton et al. (2019), who compared 4 slow-growing broiler genotypes including the genotype used in the present study.

When performance parameters were converted into economic efficiency, expressed as EBI, low, medium and high supplementation did not differ. This, however, did not account for the additional costs of the alternative riboflavin source. At the given riboflavin content of the fermentation product realized in this study (7 404 mg/kg DM) supplementation costs are estimated at approximately 9 € per ton of feed for each mg/kg feed supplemented in form of the fermentation product. The respective levels of riboflavin supplementation recommended currently by breeding companies for conventional production conditions (e.g. 8.6, 6.5 and 5.4 mg/kg for S, G and F diets of Ross broilers (Aviagen, 2019) and 9.0, 8.0 and 6.0 mg/kg for S, G and F diets of Cobb broilers (Cobb, 2018)), which do not take native contents of feed components into account, consequently lead to considerable increases of feed prices.

Based on the results of the first run, S diets with low and medium supplementation level of the fermentation product were used in the second run and riboflavin levels at low and medium level were varied in G and F diets in order to prove that the reduction of riboflavin supplementation at later fattening stages is possible without risking deficiency. First, signs of riboflavin deficiency were not observed when the fermentation product was supplemented even at low dosage in the S diet, so that results of the first run were validated. In general, growth performance was lower in the second compared with the first run, which can be explained first by a lower ME content in the G diet due to lower contents of ether extract in all diets. Specific reasons for the altered feed intake between runs could not be identified in detail. Increasing the riboflavin content in the S diet to medium level did not improve growth performance. Also similar to results of the first run, the variation of riboflavin content in G and F diets did not affect the growth performance, while feed conversion was lower in treatment D, which received riboflavin supplementation at medium level in S and at low level in G and F stages. Consequently, economic performance was superior in this group. Greater dressing percentage of treatment D at similar percentage of valuable cuts supports findings of the first run, in which MEDIUM and HIGH groups also had superior dressing but not proportion of valuable cuts. Without observing deficiency symptoms, differences in welfare indicators were expectedly not found.

In conclusion, findings confirmed results of a previous study on the use of a fermentation suspension with high native riboflavin content from fermentation of *A. gossypii* (Lambertz et al., 2019), so that also the dried product of this fermentation can be used as alternative to riboflavin produced from GMO in broiler feeding. Native contents of feed components (3.27 mg/kg DM) were not sufficient to meet the riboflavin demand, a total content of 4.50 mg/kg DM was identified as safe lower threshold. Results also proved that riboflavin levels can be reduced in grower and finisher diets without inducing riboflavin deficiency in slow-growing broilers to compensate for higher production costs of non-GMO riboflavin production.

Ethics approval

Following the Guide for the Care and Use of Agricultural Animals in Research and Teaching, animals were treated in a way avoiding any unnecessary discomfort. Birds were observed daily for behavioural and clinical abnormalities.

Data and model availability statement

The model was not deposited in an official repository.

Author ORCIDs

Christian Lambertz (corresponding author): 0000-0001-7786-4163

Author Contributions

Christian Lambertz: Conceptualization, Methodology, Software, Validation, Formal analysis, Resources, Data Curation, Writing - Original Draft, Visualization, Project administration, Funding acquisition

Jochen Leopold: Conceptualization, Methodology, Funding acquisition

Stefanie Ammer: Methodology, Software, Validation, Formal analysis, Resources, Writing - Original Draft, Visualization

Florian Leiber: Conceptualization, Methodology, Resources, Writing - Review & Editing, Project administration, Funding acquisition

Benedikt Thesing: Methodology, Software, Validation, Formal analysis, Investigation, Writing - Original Draft, Visualization

Christian Wild: Conceptualization, Methodology, Validation, Investigation, Resources, Data Curation, Writing - Review & Editing

Klaus Damme: Conceptualization, Methodology, Resources, Writing - Review & Editing, Supervision, Project administration, Funding acquisition

Declaration of Competing Interest

The authors declare that they have no competing interests.

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